the instant application. The specification is also amended to delete the inadvertently duplicated paragraphs on page 45, line 22 through page 46, line 19, that are identical to the paragraphs on page 40, lines 3-31, to correct redundancy. The specification is also amended to correct a section label. The basis for this amendment is found in the specification, in particular page 69, line 25 and page 70, line 10, which shows the chemical structures of formulas I and II in section C.4.b. The specification is also amended to add inadvertently omitted sequence identification numbers. The basis for this amendment is found in claims 6-8. The specification is also amended to add an inadvertenly omitted serial number "08/908,909". The basis for this amendment is found in the specification, in particular page 97, line 6, which incorporates the application number by reference. The specification is also amened to add the inadvertently omitted verb "are provided" to produce grammatical clarity. The specification is also amended to delete the word "which" and to add the article "the" to produce grammatical clarity.

The amendments to claims 19, 26, 40, 47, 53, 57, 70, 73, and 74 correct obvious typographical errors. The amendment to claim 47 has antecedent basis in claim 38. The amendment to claim 58 corrects a claim dependency error. The amendment to claim 64 corrects a tyographical error and finds basis in the specification, in particular, page 125, lines 18-20, and in Figure 2 which shows the association of GFP and luciferase. No new matter has been added.

Included as an attachment is a marked-up version of the specification paragraphs that are being amended, per 37 CFR §1.121.

* * *

Entry of this amendment and examination of the application are respectfully requested.

Respectfully submitted,
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Bryan *et al.*
Serial No.	09/808,898
Filed:	March 15, 2001
For:	*RENILLA RENIFORMIS FLUORESCENT PROTEINS AND TEH USE THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY ITEMS*
Art Unit:	Unassigned
Examiner:	Unassigned

ATTACHMENT TO THE PRELIMINARY AMENDMENT
MARKED UP PARAGRAPHS AND CLAIMS (37 CFR §1.121)

IN THE SPECIFICATION:

Please amend the specification as follows:

Please amend the paragraph on page 2, lines 8-24, as follows:

BACKGROUND OF THE INVENTION

Luminescence is a phenomenon in which energy is specifically channeled to a molecule to produce an excited state. Return to a lower energy state is accompanied by release of a photon (hγ). Luminescence includes fluorescence, phosphorescence, chemiluminescence and bioluminescence. Bioluminescence is the process by which living organisms emit light that is visible to other organisms. Luminescence may be represented as follows:

$$A + B \rightarrow X^* + Y$$

$$X^* \rightarrow X + h\nu,$$

where X^* is an electronically excited molecule and hγ represents light emission upon return of X^* to a lower energy state. Where the luminescence is bioluminescence, and creation of the excited state <u>is</u> [derives] <u>derived</u> from an enzyme catalyzed reaction. The color of the emitted light in a bioluminescent (or chemiluminescent or other luminescent) reaction is characteristic of the excited molecule, and is independent from its source of excitation and temperature.

Please amend the paragraph on page 3, lines 6-15, as follows:

Though rare overall, bioluminescence is more common in marine organisms than in terrestrial organisms. Bioluminescence has developed from as many as thirty evolutionarily distinct origins and, thus, is manifested in a variety of ways so that the biochemical and physiological mechanisms responsible for bioluminescence in different organisms are distinct. Bioluminescent species span many genera and include microscopic organisms, such as bacteria (primarily marine bacteria including *Vibrio* species), fungi, algae and dinoflagellates, to marine organisms, including arthropods, mollusks, echinoderms, and chordates, and terrestrial [organism] organisms including annelid worms and insects.

Please amend the paragraph on page 5, lines 5-11, as follows:

Because of the utility of luciferases as reagents in analytical systems and the potential for use in high throughput screening systems, there is a need to identify and [isolated] isolate a variety of luciferases that have improved or different spectral properties compared to those presently available. For all these reasons, it would be advantageous to have luciferases from a variety of species, such as *Gaussia* and various *Renilla* species available.

Please amend the paragraphs beginning on page 5, line 30, through page 6, line 24, as follows:

The best characterized GFPs are those isolated from the jellyfish species *Aequorea*, particularly *Aequorea victoria* (*A. victoria*) and *Aequorea forskålea* (Ward *et al.* (1982) *Biochemistry 21*:4535-4540; Prendergast *et al.* (1978) *Biochemistry 17*:3448-3453). Purified *A. victoria* GFP is a monomeric protein of about 27 [Kda] kDa that absorbs blue light with excitation wavelength maximum of 395 nm, with a minor peak at 470 nm, and emits green fluorescence with an emission wavelength of about 510 nm and a minor peak near 540 nm (Ward *et al.* (1979) *Photochem. Photobiol. Rev 4*:1-57). This GFP has certain limitations.

The excitation maximum of the wildtype GFP is not within the range of wavelengths of standard fluorescein detection optics.

The detection of green fluorescence does not require any exogenous substrates or co-factors. Instead, the high level of fluorescence results from the intrinsic chromophore of the protein. The chromophore includes modified amino acid residues within the polypeptide chain. For example, the fluorescent chromophore of *A. victoria* GFP is encoded by the hexapeptide sequence, FSYGVQ, encompassing amino acid residues 64-69. The chromophore is formed by the intramolecular cyclization of the polypeptide backbone at residues Ser65 and Gly67 and the oxidation of the α-β bond of residue Tyr66 (*e.g.*, see Cody *et al.* (1993) *Biochemistry 32*:1212-1218; Shimomura (1978) *FEBS Letters 104*:220-222; Ward *et al.* (1989) *Photochem. Photobiol. 49*:62S). The emission spectrum of the isolated chromophore and the denatured protein at neutral [Ph] pH do not match the spectrum of the native protein, suggesting that chromophore formation occurs post-translationally (*e.g.*, see Cody *et al.* (1993) *Biochemistry 32*:1212-1218).

Please amend the paragraphs beginning on page 7, line 1, through page 8, line 3, as follows:

DNA encoding an isotype of *A. victoria* GFP has been isolated and its nucleotide sequence has been determined (*e.g.*, see Prasher (1992) *Gene 111*:229-233). The *A. victoria* [CDNA] cDNA contains a 714 nucleotide open reading frame that encodes a 238 amino acid polypeptide of a calculated M_r of 26,888 Da. Recombinantly expressed *A. victoria* GFPs retain their ability to fluoresce *in vivo* in a wide variety organisms, including bacteria (*e.g.*, see Chalfie *et al.* (1994) *Science 263*:802-805; Miller *et al.* (1997) *Gene 191*:149-153), yeast and fungi (Fey *et al.* (1995) *Gene 165*:127-130; Straight *et al.* (1996) *Curr. Biol. 6*:1599-1608; Cormack *et al.* (1997) *Microbiology 143*:303-311), *Drosophila* (*e.g.*, see Wang *et al.* (1994) *Nature 369*:400-403; Plautz (1996) *Gene 173*:83-87), plants (Heinlein *et al.* (1995); Casper *et al.* (1996)

Gene 173:69-73), fish (Amsterdam *et al.* (1995)), and mammals (Ikawa *et al.* (1995). *Aequorea* GFP vectors and isolated *Aequorea* GFP proteins have been used as markers for measuring gene expression, cell migration and localization, microtubule formation and assembly of functional ion channels (*e.g.*, see Terry *et al.* (1995) *Biochem. Biophys. Res. Commun. 217*:21-27; Kain *et al.* (1995) *Biotechniques 19*:650-655)._The *A. victoria* GFP, however, is not ideal for use in analytical and diagnostic processes. Consequently GFP mutants have been selected with the hope of identifying mutants that have single excitation spectral peaks shifted to the red.

In fact a stated purpose in constructing such mutants has been to attempt to make the *A. victoria* GFP more like the GFP from *Renilla*, but which has properties that make it far more ideal for use as an analytical tool. For many practical applications, the spectrum of [Renilla] <u>*Renilla*</u> GFP is [be] preferable to that of the *Aequorea* GFP, because wavelength discrimination between different fluorophores and detection of resonance energy transfer are easier if the component spectra are tall and narrow rather than low and broad (see, U.S. Patent No. 5,625,048). Furthermore, the longer wavelength excitation peak (475 nm) of *Renilla* GFP is almost ideal for fluorescein filter sets and is resistant to photobleaching, but has lower amplitude than the shorter wavelength peak at 395 nm, which is more susceptible to photobleaching (Chalfie *et al.* (1994) *Science 263*:802-805).

Please amend the paragraphs beginning on page 9, line 29, through page 10, line 27, as follows:

The bioluminescence-generating system includes, in addition to the luciferase, a *Renilla reniformis* [ ] GFP or mutated form thereof. These compositions can be used in a variety of methods and systems, such as <u>those</u> included in conjunction with diagnostic systems for the *in vivo* detection of neoplastic tissues and other tissues, such as those methods described herein.

Combinations of the *Renilla reniformis* GFP with [an] articles of manufacture to produce novelty items are provided. These novelty items are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as cosmetics, bath powders, body lotions, gels, powders and creams, nail polishes, make-up, toothpastes and other dentifrices, soaps, body paints, and bubble bath; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable form. The combinations optionally include a bioluminescence generating system. The bioluminescence generating systems can be provided as two compositions: a first composition containing a luciferase and a second composition containing one or more additional components of a bioluminescence generating system.

Please amend the paragraphs on page 11, lines 7-27, as follows:

GFPS

Isolated nucleic acids that encode GFP from *Renilla reniformis* are provided herein. Also provided are isolated and purified nucleic acids that encode a component of the bioluminescence generating system and [a] the green fluorescent protein (GFP) (see SEQ ID Nos. 23-27). In particular, nucleic acid molecules that encode *Renilla reniformis* green fluorescent protein (GFPs)

and nucleic acid probes and primers derived therefrom are provided. Nucleic acid molecules encoding *Renilla reniformis* GFP are provided (see SEQ ID Nos. 23-26).

Nucleic acid probes and primers containing 14, 16, 30, 100 or more contiguous nucleotides from any of SEQ ID Nos. 23-26 are provided. Nucleic acid probes can be labeled, [which] if needed, for detection, containing at least about 14, preferably at least about 16, or, if desired, 20 or 30 or more, contiguous nucleotides [of]or sequence of nucleotides encoding the *Renilla reniformis* GFP.

Methods using the probes for the isolation and cloning of GFP-encoding DNA in *Renilla reniformis* are also provided. Vectors containing DNA encoding the *Renilla reniformis* GFP are provided. In particular, expression vectors that contain DNA encoding a *Renilla reniformis* or in operational association with a promoter element that allows for the constitutive or inducible expression of *Renilla reniformis* are provided.

Please amend the paragraph beginning on page 12, line 19, through page 13, line 3, as follows:

Non-radioactive energy transfer reactions, such as FET or FRET, FP and HTRF assays, are homogeneous luminescence assays based on energy transfer and are carried out between a donor luminescent label and an acceptor label (see, *e.g.*, Cardullo *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*:8790-8794; Peerce *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*:8092-8096; U.S. Patent No. 4,777,128; U.S. Patent No. 5,162,508; U.S. Patent No. 4,927,923; U.S. Patent No. 5,279,943; and International PCT Application No. WO 92/01225). Non-radioactive energy transfer reactions using GFPs have been developed (see, International PCT application Nos. WO 98/02571 and WO 97/28261). Non-

radioactive energy transfer reactions using GFPs and luciferases, such as a luciferase and its cognate GFP (or multimers thereof), such as in a fusion protein, are contemplated herein.

Please amend the paragraph beginning on page 13, line 11, through page 14, line 4, as follows:

The nucleic acids provide an opportunity to produce luciferases and GFPs, which have advantageous application in all areas in which luciferase/luciferins and GFPs have application. The nucleic acids can be used to obtain and produce GFPs and GFPs from other, particularly *Renilla*, species using the probes described herein that correspond to conserved regions. These GFPs have advantageous application in all areas in which GFPs and/or luciferase/luciferins have application. For example, [The]the [GFP's] GFPs provide a means to amplify the output signal of bioluminescence generating systems. *Renilla* GFP has a single excitation absorbance peak in blue light (and around 498 nm) and a predominantly single emission peak around 510 nm (with a small shoulder near 540). This [spectra]spectrum provides a means for it to absorb blue light and efficiently convert it to green light. This results in an amplification of the output. When used in conjunction with a bioluminescence generating system that yields blue light, such as *Aequorea* or *Renilla* or *Vargula* (*Cypridina*), the output signal for any application, including diagnostic applications, is amplified. In addition, this green light can serve as an energy donor in fluorescence-based assays, such as fluorescence polarization assays, FET (fluorescent energy transfer) assays, FRET (fluorescent resonance energy transfer) assays and HTRF (homogeneous time-resolved fluorescence) assays. Particular assays, herein referred to as BRET (bioluminescence

resonance energy transfer assays in which energy is transferred from a bioluminescence reaction of a luciferase to a fluorescent protein), are provided.

Please amend the paragraph beginning on page 14, line 21, through page 15, line 5, as follows:

The DNA may be introduced as a linear DNA molecule (fragment) or may be included in an expression vector for stable or transient expression of the encoding DNA. In certain embodiments, the cells that contain DNA or RNA encoding a *Renilla* GFP also express the recombinant *Renilla* GFP or polypeptide. It is preferred that the cells are selected to express functional GFPs that retain the ability to fluorescence and that are not toxic to the host cell. In some embodiments, cells may also include heterologous nucleic acid encoding a component of a bioluminescence-generating system, preferably a photoprotein or luciferase. In preferred embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species *Aequorea*, *Vargula*, *Pleuromamma*, *Ptilosarcus* or *Renilla*. In more preferred embodiments, the bioluminescence-generating system component is a *Renilla reniformis* luciferase or *mulleri* including the amino acid sequence set forth in SEQ ID No. 18 or the *Pleuromamma* luciferase set forth in SEQ ID No. 28, or the *Gaussia* luciferase set forth in SEQ ID No. 19.

Please amend the paragraphs beginning on page 16, line 21, through page 18, line 13, as follows:

Compositions that contain a *Renilla reniformis* GFP or GFP peptide and at least one component of a bioluminescence-generating system, preferably a luciferase, luciferin or a luciferase and a luciferin, are provided. In preferred embodiments, the luciferase/luciferin [bioluminescence- generating] bioluminescence-generating system is selected from those isolated from: an insect system, a coelenterate system, a ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an

earthworm system. [Bioluminescence- generating] Bioluminescence-generating systems include those isolated from *Renilla*, *Aequorea*, and *Vargula*, *Gaussia* and *Pleuromamma*.

Combinations containing a first composition containing a *Renilla reniformis* GFP or *Ptilosarcus* GFP or mixtures thereof and a second composition containing a [bioluminescence- generating] bioluminescence-generating system for use with inanimate articles of manufacture to produce novelty items are provided. These novelty items, which are articles of manufacture, are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as bath powders, body lotions, gels, powders and creams, nail polishes, cosmetics including make-up, toothpastes and other dentifrices, soaps, cosmetics, body paints, and bubble bath, bubbles made from non-detergent sources, particularly proteins such as albumin and other non-toxic proteins; in fishing lures and glowing transgenic worms, particularly crosslinked polyacrylamide containing a fluorescent protein and/or components of a bioluminescence generating system, which glow upon contact with water; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic animals, such as transgenic fish, worms, monkeys, rodents, ungulates, ovine, ruminants and others, that express a luciferase and/or *Renilla reniformis* GFP; transgenic worms that express *Renilla reniformis* GFP and are used as lures; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that express luciferase and *Renilla reniformis* GFP, transgenic plants that express *Renilla reniformis* GFP, particularly ornamental plants, such as orchids, roses, and other plants with decorative flowers; transgenic plants and animals in which the *Renilla reniformis* GFP is a marker for tracking introduction of other genes;

and beverages, such as beer, wine, champagne, soft drinks, milk and ice cubes and ice in other configurations containing *Renilla reniformis* GFP; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable [form]forms.

Any article of manufacture that can be combined with a bioluminescence-generating system and *Renilla reniformis* GFP or with just a *Renilla reniformis* GFP, as provided herein, that thereby [provide]provides entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce, such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles.

Please amend the paragraph on page 21, lines 1-12, as follows:

Methods for generating chimeric GFP fusion proteins are provided. The methods include linking DNA encoding a gene of interest, or portion thereof, to DNA encoding a GFP coding region in the same translational reading frame. The encoded-protein of interest may be linked in-frame to the amino- or carboxyl-terminus of the GFP. The DNA encoding the chimeric protein is then linked in operable association with a promoter element of a suitable expression vector. Alternatively, the promoter element can be obtained directly from the targeted gene of interest and the promoter-containing fragment linked upstream of the GFP coding sequence to produce chimeric GFP proteins or [two]to produce polycistronic mRNAs that encode the *Renilla reniformis* GFP and a luciferase, preferably a *Renilla* luciferase, more preferably *Renilla reniformis* luciferase.

Please amend the paragraph beginning on page 21, line 28, through page 22, line 5, as follows:

Kits containing the GFPs for use in the methods, including those described herein, are provided. In one embodiment, the kits containing an article of manufacture and appropriate reagents for generating bioluminescence are provided. The kits containing such soap compositions, with preferably a moderate [Ph]pH (between 5 and 8) and bioluminescence generating reagents, including luciferase and luciferin and the GFP are provided herein. These kits, for example, can be used with a bubble-blowing or producing toy. These kits can also include a reloading or charging cartridge or can be used in connection with a food.

Please amend the paragraph beginning on page 22, line 19, through page 23, line 2, as follows:

In other embodiments, the kits are used for detecting and identifying diseases, particularly infectious diseases, using multi-well assay devices and include a multi-well assay device containing a plurality of wells, each having an integrated photodetector, to which an antibody or panel of antibodies specific for one or more infectious agents are attached, and composition containing a secondary antibody, such as an antibody specific for the infectious agent that is linked to a *Renilla reniformis* GFP protein, a chimeric antibody-*Renilla reniformis*[)] GFP fusion protein or $F(Ab)_2$ antibody fragment-*Renilla reniformis* GFP fusion protein. A second composition [containing]contains a bioluminescence generating system that emits a wavelength of light within the excitation range of the *Renilla mulleri* GFP, such as species of *Renilla* or *Aequorea*, for exciting the *Renilla reniformis*, which produces light that is detected by the photodetector of the device to indicate the presence of the agent.

Please amend the paragraph on page 24, lines 4-14, as follows:

[FIGURE 2]FIGURES 2A-D [illustrates]illustrate the underlying principle of Bioluminescent Resonance Energy Transfer (BRET) and its use as sensor: A) in isolation, a luciferase, preferably an anthozoan luciferase, emits blue light from the coelenterazine-derived chromophore; B) in isolation, a GFP, preferably an anthozoan GFP that binds to the luciferase, that is excited with blue-green light emits green light from its integral peptide based fluorophore; C) when the luciferase and GFP associate as a complex *in vivo* or *in vitro*, the luciferase non-radiatively transfers its reaction energy to the GFP flurophore, which then emits the green light; D) any molecular interaction that disrupts the luciferase-GFP complex can be quantitatively monitored by observing the spectral shift from green to blue light.

Please amend the paragraphs on page 24, lines 16-26, as follows:

FIGURE 4 depicts the substitution of altered fluorophores into the background of *Ptilosarcus*, *Renilla mulleri* and *Renilla reniformis* GFPs (the underlined regions correspond[s] to amino acids 56-75 of SEQ ID No. 27 *Renilla reniformis* GFP; amino acids 59-78 of SEQ ID No. 16 *Renilla mulleri* GFP; and amino acids 59-78 of SEQ ID No. 32 for *Ptilosarcus* GFP).

FIGURE 5 depicts the three anthozoan fluorescent [protein]proteins for which a crystal structure exists[,]; another is available commercially from Clontech as dsRed (from *Discosoma striata*; also known as drFP583, as in this alignment); a dark gray background depicts amino acid conservation, and a light gray background depicts shared physicochemical properties.

Please amend the paragraphs beginning on page 34, line 25, through page 35, line 5, as follows:

As used herein, a nucleic acid probe is single-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases, preferably at least 16 contiguous bases, typically about 30, that are the same as

(or the complement of) any 14 or more contiguous bases set forth in any of SEQ ID [No.]Nos. 23-25 and herein. Among the preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode regions that are conserved among *Renilla* species. Probes from regions conserved among *Renilla* species GFPs are for isolating GFP-encoding nucleic acid from *Renilla* libraries.

In preferred embodiments, the nucleic acid probes are degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides[, are provided].

Please amend the paragraphs on page 36, lines 6-29, as follows:

As used herein, operatively linked or operationally associated refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, [potential]potentially inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak (1991) *J. Biol. Chem. 266*:19867-19870) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, to target a targeted agent, such as a luciferase, means to direct it to a cell that expresses a selected receptor or other cell surface protein by linking the agent to a such agent. Upon binding to or interaction with the receptor or cell surface protein, the targeted[,] agent can be reacted with an

appropriate substrate and activating agents, whereby bioluminescent light is produced and the tumorous tissue or cells distinguished from non-tumorous tissue.

Please amend the paragraph on page 39, lines 1-3, as follows:

As used herein, targeting agent (TA) refers to an agent that specifically or preferentially targets a linked targeted agent, a luciferin or luciferase, to a neoplastic cell or tissue.

Please amend the paragraphs beginning on page 45, line 22, through page 46, line 19, as follows:

[Examples of receptors and applications using such receptors, include but are not restricted to:

a) enzymes: specific transport proteins or enzymes essential to survival of microorganisms, which could serve as targets for antibiotic (ligand) selection;

b) antibodies: identification of a ligand-binding site on the antibody molecule that combines with the epitope of an antigen of interest may be investigated; determination of a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for auto-immune diseases

c) nucleic acids: identification of ligand, such as protein or RNA, binding sites;

d) catalytic polypeptides: polymers, preferably polypeptides, that are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products; such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, in which the functionality is

capable of chemically modifying the bound reactant (see, *e.g.*, U.S. Patent No. 5,215,899);

e) hormone receptors: determination of the ligands that bind with high affinity to a receptor is useful in the development of hormone replacement therapies; for example, identification of ligands that bind to such receptors may lead to the development of drugs to control blood pressure; and

f) opiate receptors: determination of ligands that bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.]

Please amend the paragraph on page 48, lines 1-11, as follows:

B. FLUORESCENT PROTEINS

The GFP from *Aequorea* and that of the sea pansy *Renilla reniformis* share the same chromophore, yet Aequorea GFP has two absorbance peaks at 395 and 475 nm, whereas [Renilla] *Renilla* GFP has only a single absorbance peak at 498 nm, with about 5.5 fold greater monomer extinction coefficient than the major 395 nm peak of the Aequorea protein (Ward, W. W. in [Biohtminescence]Bioluminescence and Chemiluminescence (eds. DeLuca, M. A. & McElroy, W. D.) 235-242 (Academic Press, New York, 1981)). The spectra of the isolated chromophore and denatured protein at neutral pH do not match the spectra of either native protein (Cody, C. W. *et al.* (1993) *Biochemistry 32*:1212-1218).

Please amend the paragraph beginning on page 49, line 22, through page 51, line 6, as follows:

In particular, nucleic acid molecules encoding a *Renilla reniformis* GFP having any of the following sequences are provided (see SEQ ID Nos. 23-25):

[Renilla renformis] *Renilla reniformis* GFP Clone-1

GGCACGAGGGTTTCCTGACACAATAAAAACCTTTCAAATTGTTTCTC
TGTAGCAGTAAGTATGGATCTCGCAAAACTTGGTTTGAAGGAAGTG
ATGCCTACTAAAATCAACTTAGAAGGACTGGTTGGCGACCACGCTT

TCTCAATGGAAGGAGTTGGCGAAGGCAACATATTGGAAGGAACTCA
AGAGGTGAAGATATCGGTAACAAAAGGCGCACCACTCCCATTCGC
ATTTGATATCGTATCTGTGGCTTTTTCATATGGGAACAGAGCTTA
TACCGGTTACCCAGAAGAAATTTCCGACTACTTCCTCCAGTCGTT
TCCAGAAGGCTTTACTTACGAGAGAAACATTCGTTATCA
AGATGGAGGAACTGCAATTGTTAAATCTGATATAAGCTTGGAA
GATGGTAAATTCATAGTGAATGTAGACTTCAAAGCGAAGGATCT
ACGTCGCATGGGACCAGTCATGCAGCAAGACATCGTGGGTATGCA
GCCATCGTATGAGTCAATGTACACCAATGTCACTTCAGTTATAGGGGA
ATGTATAATAGCATTCAAACTTCAAACTGGCAAGCATTTCACTTACCA
CATGAGGACAGTTTACAAATCAAAGAAGCCAGTGGAAACTATGCCA
TTGTATCATTTCATCCAGCATCGCCTCGTTAAGACCAATGTGGACA
CAGCCAGTGGTTACGTTGTGCAACACGAGACAGCAATTGCAGCGCATTCTACA
ATCAAAAAAATTGAAGGCTCTTTACCATAGATACCTGTACACAATTATTCTATG
CACGTAGCATTTTTTTGGAAATATAAGTGGTATTGTTCAATAAAATATTAAATA
TAAAAAAAAAAAAAAAAAAAAAA;

[Renilla renformis] *Renilla reniformis* GFP Clone-2

GGCACGAGGCTGACACAATAAAAAACCTTTCAAATTGTTTCTCTGTAGCAGGA
AGTATGGATCTCGCAAAACTTGGTTTGAAGGAAGTGATGCCTACTAAAATCAA
CTTAGAAGGACTGGTTGGCGACCACGCTTTCTCAATGGAAGGAGTTGGCGAAG
GCAACATATTGGAAGGAACTCAAGAGGTGAAGATATCGGTAACAAAAGGCGC
ACCACTCCCATTCGCATTTGATATCGTATCTGTTGCTTTCTCATATGGGAACAG
AGCTTATACTGGTTACCCAGAAGAAATTTCCGACTACTTCCTCCAGTCGTTTCC
AGAAGGCTTTACTTACGAGAGAAACATTCGTTATCAAGATGGAGGAACTGCAA
TTGTTAAATCTGATATAAGCTTGGAAGATGGTAAATTCATAGTGAATGTAGACT
TCAAAGCGAAGGATCTACGTCGCATGGGACCAGTCATGCAGCAAGACATCGT
GGGTATGCAGCCATCGTATGAGTCAATGTACACCAATGTCACTTCAGTTATAG
GGGA
ATGTATAATAGCATTCAAACTTCAAACTGGCAAACATTTCACTTACCACATGA
GGACAGTTTACAAATCAAAGAAGCCAGTGGAAACTATGCCATTGTATCATTTC
ATCCAGCATCGCCTCGTTAAGACCAATGTGGACACAGCCAGTGGTTACGTTGT
GCAACACGAGACAGCAATTGCAGCGCATTCTACAATCAAAAAAATTGAAGGC
TCTTTACCATAGATATCTATACACAATTA
TTCTATGCACGTAGCATTTTTTTTGGAAATATAAGTGGTATTGTTCAATAAAATA
TTAAATATAAAAAAAAAAAAAAAAAAAAAA; and

[Renilla renformis] *Renilla reniformis* GFP Clone-3

GGCACGAGGGTTTCCTGACACAATAAAAACCTTTCAAATTGTTTCTCTGTAGC
AGTAAGTATGGATCTCGCAAAACTTGGTTTGAAGGAAGTGATGCCTACTAAAA
TCAACTTAGAAGGACTGGTTGGCGACCACGCTTTCTCAATGGAAGGAGTTGGC
GAAGGCAACATATTGGAAGGAACTCAAGAGGTGAAGATATCGGTAACAAAAG
GCGCACCACTCCCATTCGCATTTGATATCGTATCTGTGGCTTTTTCATATGGGA

ACAGAGCTTATACCGGTTACCCAGAAGAAATTTCCGACTACTTCCTCCAGTCG
TTTCCAGAAGGCTTTACTTACGAGAGAAACATTCGTTATCAAGATGGAGGAAC
TGCAATTGTTAAATCTGATATAAGCTTGGAAGATGGTAAATTCATAGTGAATGT
AGACTTCAAAGCGAAGGATCTACGTCGCATGGGACCAGTCATGCAGCAAGAC
ATCGTGGGTATGCAGCCATCGTATGAGTCAATGTACACCAATGTCACTTCAGT
TATAGGGGAATGTATAATAGCATTCAAACTTCAAACTGGCAAGCATTTCACTT
ACCACATGAGGACAGTTTACAAATCAAAGAAGCCAGTGGAAACTATGCCATT
GTATCATTTCATCCAGCATCGCCTCGTTAAGACCAATGTGGACACAGCCAGTG
GTTACGTTGTGCAACACGAGACAGCAATTGCAGCGCATTCTACAATCAAAAAA
ATTGAAGGCTCTTTACCATAGATACCTGTACACAATTA
TTCTATGCACGTAGCATTTTTTTGGAAATATAAGTGGTATTGTTCAATAAAATA
TTAAATATATGCTTTTGCAAAAAAAAAAAAAAAAAAAAA

[are provided].

Please amend the paragraph on page 51, lines 10-17, as follows:

Also contemplated are the coding portion of the sequence of nucleotides that hybridize under moderate or high stringency to the sequence of nucleotides set forth above[.], particularly when using probes provided herein[, are provided]. Probes derived from this nucleic acid that can be used in methods provided herein to [isolated]isolate GFPs from any *Renilla reniformis* species are provided. In an exemplary embodiment, nucleic acid encoding *Renilla reniformis* GFP is provided. This nucleic acid encodes the sequence of amino acids set forth above.

Please amend the paragraph on page 61, lines 3-9, as follows:

Thus, for example, in presence of calcium, 5 mg of luciferin, such as coelenterazine, in one liter of water will glow brightly for at least about 10 to 20 minutes, depending on the temperature of the water, when about 10 [mgs]mg of luciferase, such as aequorin photoprotein luciferase or luciferase from *Renilla*, is added thereto. Increasing the concentration of luciferase, for example, to 100 mg/l, provides a particularly brilliant display of light.

Please amend the paragraph beginning on page 61, line 30, through page 62, line 7, as follows:

Renilla luciferase and the other coelenterate and ctenophore luciferases, such as the aequorin photoprotein, use imidazopyrazine substrates, particularly the substrates generically called coelenterazine (see, formulae (I) and (II) of Section [B.1.b, above]C.4.b, below). Other genera that have luciferases that use a coelenterazine include: squid, such as *Chiroteuthis*, *Eucleoteuthis*, *Onychoteuthis*, *Watasenia*, cuttlefish, *Sepiolina*; shrimp, such as *Oplophorus*, *Acanthophyra*, *Sergestes*, and *Gnathophausia*; deep-sea fish, such as *Argyropelecus*, *Yarella*, *Diaphus*, *Gonadostomias* and *Neoscopelus*.

Please amend the paragraph on page 63, lines 12-21, as follows:

In certain embodiments herein, about 1 to 10 mg, or preferably 2-5 mg, more preferably about 3 mg of coelenterazine will be used with about 100 mg of *Renilla* luciferase. The precise amounts, of course can be determined empirically, and, also will depend to some extent on the ultimate concentration and application. In particular, [about] addition of about 0.25 ml of a crude extract from the bacteria that express *Renilla* to 100 ml of a suitable assay buffer and about 0.005 μg was sufficient to produce a visible and lasting glow (see, U.S. Patent Nos. 5,418,155 and 5,292,658, which describe recombinant production of *Renilla reniformis* luciferase).

Please amend the paragraph on page 72, lines 19-25, as follows:

The systems, such as the *Vargula* bioluminescent systems, are particularly preferred herein because the components are stable at room temperature if dried and powdered and will continue to react even if contaminated. Further, the bioluminescent reaction requires only the luciferin/luciferase components in concentrations as low as 1:40 parts per billion to 1:100 parts per billion, water and molecular oxygen to proceed. An exhausted system can be renewed by addition of luciferin.

Please amend the paragraphs beginning on page 76, lin 6, through page 77, line 7, as follows:

Modified luciferases [that]can generate light at different wavelengths (compared with native luciferase), and thus, may be selected for their color-producing characteristics. For example, synthetic mutant beetle luciferase(s) and DNA encoding such luciferases that produce bioluminescence at a wavelength different from wild-type luciferase are known (Promega Corp, International PCT Application No. WO 95/18853, which is based on U.S. application Serial No. 08/177,081). The mutant beetle luciferase has an amino acid sequence differing from that of the corresponding wild-type *Luciola cruciata* (see, *e.g.*, U.S. Patent Nos. 5,182,202, 5,219,737, 5,352,598, see, also SEQ ID No.3) by a substitution(s) at one or two positions. The mutant luciferase produces a bioluminescence with a wavelength of peak intensity that differs by at least 1 nm from that produced by wild-type luciferases.

Other mutant luciferases can be produced. Mutant luciferases with the amino acid sequence of wild-type luciferase, but with at least one mutation in which valine is replaced by isoleucine at the amino acid number 233, valine by isoleucine at 239, serine by asparagine at 286, glycine by serine at 326, histidine by tyrosine at 433 or proline by serine at 452 are known (see, e.g., U.S. Patent Nos. 5,219,737, and 5,330,906). The luciferases are produced by expressing DNA-encoding each mutant luciferase in *E. coli* and isolating the protein. These luciferases produce light with colors that differ from wild-type. The mutant luciferases catalyze luciferin to produce red (λ 609 nm and 612 nm), orange(λ595 and 607 nm) or green (λ 558 nm) light. The other physical and chemical properties of mutant luciferase are substantially identical to native wild type-luciferase. The mutant luciferase has the amino acid sequence of *Luciola cruciata* luciferase with an alteration selected from Ser 286 replaced by Asn, Gly 326 replaced by Ser, His 433 replaced by Tyr or Pro 452 replaced by Ser.

Thermostable luciferases are also available (see, *e.g.*, U.S. Patent No. 5,229,285; see, also International PCT Application No. WO 95/25798, which provides *Photinus* luciferase in which the glutamate at position 354 is replaced with lysine and *Luciola* luciferase in which the glutamate at 356 is replaced with lysine).

Please amend the paragraph beginning on page 79, line 19, through page 80, line 2, as follows:

(1) Luciferases

Bacterial luciferase, as exemplified by luciferase derived from *Vibrio harveyi* (EC 1.14.14.3, alkanol reduced-FMN-oxygen oxidoreductase 1-hydroxylating, luminescing), is a mixed function oxidase, formed by the association of two different protein subunits α and β. The α-subunit has an apparent molecular weight of approximately 42,000 [kD]kDa and the β-subunit has an apparent molecular weight of approximately 37,000 [kD]kDa (see, *e.g.*, Cohn *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A. 90*:102-123). These subunits associate to form a 2-chain complex luciferase enzyme, which catalyzes the light emitting reaction of bioluminescent bacteria, such as *Vibrio harveyi* (U.S. Patent No. 4,581,335; Belas *et al.* (1982) *Science 218*:791-793), *Vibrio fischeri* (Engebrecht *et al.* (1983) *Cell 32*:773-781; Engebrecht *et al.* (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*:4154-4158) and other marine bacteria.

Please amend the paragraph on page 87, lines 9-19, as follows:

3. Cloning of *Renilla reniformis* Green Fluorescent Protein

Renilla reniformis GFP has 233 amino acids compared to GFPs from animals that contain luciferase-GFP bioluminescent systems *Renilla mulleri*, *Ptilosarcus* and *Aequorea Victoria*. Other such GFPs have 238 amino acids. At the amino acid level, *Renilla reniformis* is respectively 53, 51 and 19% identical to the GFPs from these animals. The extent of identity of *Renilla reniformis* GFP to the half dozen cloned anthozoan coral GFPs, which do not contain associated

luciferases, ranges from 32 to 38%. The overall identity among these GFPs is surprisingly low for a protein evolved from a common ancestor. These relationships are depicted as a phylogenetic tree (Figure 1).

Please amend the paragraph on page 92, lines 1-8, as follows:

Host organisms include those organisms in which recombinant production of heterologous proteins have been carried out, such as, but not limited to, bacteria (for example, *E. coli*), yeast (for example, *Saccharomyces cerevisiae* and *Pichia pastoris*), fungi, baculovirus/insect systems, amphibian cells, mammalian cells, plant cells and insect cells. Presently preferred host organisms are strains of bacteria or yeast. Most preferred host organisms are strains of *E. coli* or *Saccharomyces cerevisiae*.

Please amend the paragraph on page 93, lines 4-9, as follows:

5. Recombinant cells expressing heterologous nucleic acid encoding Renilla GFP

Cells, vectors and methods are described with respect to *Renilla*. The same cells, vectors and methods may be used for expressing luciferases and other GFPs from species including *Gaussia*, *Pleuromamma* and *Ptilosarcus*.

Please amend the paragraph on page 95, lines 19-28, as follows:

Compositions that contain a *Renilla mulleri* GFP or GFP peptide dand at least one component of a bioluminescence-generating system, preferably a luciferase, luciferin or a luciferase and a luciferin, are provided. In preferred embodiments, the luciferase/luciferin [bioluminescence- generating] bioluminescence-generating system is selected from those isolated from: an insect system, a coelenterate system, a ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an

earthworm system. Presently preferred bioluminescence-generating systems are those isolated from *Renilla*, *Aequorea*, and *Vargula*.

Please amend the paragraphs beginning on page 96, line 23, through page 97, line 12, as follows:

In other embodiments, the *Renilla* luciferase and the remaining components may be packaged as separate compositions, that, upon mixing, glow. For example, a composition containing *Renilla* luciferase may be provided separately from, and for use with, [an] a separate composition containing a bioluminescence substrate and bioluminescence activator. In another instance, luciferase and luciferin compositions may be separately provided and the bioluminescence activator may be added after, or simultaneously with, mixing of the other two compositions.

3. Conjugates

Conjugates are provided herein for a variety of uses. Among them [arer] are for targeting to tumors for visualization of the tumors, particularly *in situ* during surgery. A general description of these conjugates and the uses thereof is described in allowed U.S. application Serial No. 08/908,909. In practice, prior to a surgical procedure, the conjugate is administered via any suitable route, whereby the targeting agent binds to the targeted tissue by virtue of its specific interaction with a tissue-specific cell surface protein. During surgery the tissue is contacted, with the remaining component(s), typically by spraying the area or local injection, and any tissue to which conjugate is bound will glow. The glow should be sufficient to see under dim light or, if necessary, in the dark.

Please amend the paragraph on page 102, lines 16-24, as follows:

c. Anti-tumor Antigen Antibodies

Polyclonal and monoclonal antibodies may be produced against selected antigens. Alternatively, many such antibodies are presently available. An

exemplary list of antibodies and the tumor antigen for which each has been directed against is provided in U.S. application Serial No. 08/908,909, which is incorporated by reference in its entirety. It is contemplated that any of the antibodies listed may be conjugated with a bioluminescence generating component following the methods provided herein.

Please amend the paragraphs beginning on page 115, line 28, through page 116, line 31, as follows:

In one embodiment, the chip is made using an integrated circuit with an array, such as an X-Y array, of photodetectors, such as that described in co-pending U.S. application Serial No. 08/990,103. The surface of circuit is treated to render it inert to conditions of the diagnostic assays for which the chip is intended, and is adapted, such as by derivatization for linking molecules, such as antibodies. A selected antibody or panel of antibodies, such as an antibody specific for particularly bacterial antigen, is affixed to the surface of the chip above each photodetector. After contacting the chip with a test sample, the chip is contacted with a second antibody linked to the GFP, such as the *Renilla* GFP, to form a chimeric [antibody- GFP] antibody-GFP fusion protein or an antibody linked to a component of a bioluminescence generating system, such as a *Pleuromamma*, *Gaussia* or *R. mulleri* luciferase. The antibody is specific for the antigen. The remaining components of the bioluminescence generating reaction are added, and, if any of the antibodies linked to a component of a bioluminescence generating system are present on the chip, light will be generated and detected by the adjacent photodetector. The photodetector is operatively linked to a computer, which is programmed with information identifying the linked antibodies, records the event, and thereby identifies antigens present in the test sample.

3. Methods for generating *Renilla mulleri* luciferase, *Pleuromamma* luciferase and *Gaussia* luciferase fusion proteins with *Renilla reniformis* GFP.

Methods for generating GFP and luciferase fusion proteins are provided. The methods include linking DNA encoding a gene of interest, or portion thereof, to DNA encoding a *Renilla reniformis* GFP and a luciferase in the same translational reading frame. The encoded-protein of interest may be linked in-frame to the amino- or carboxyl-terminus of the GFP or luciferase. The DNA encoding the chimeric protein is then linked in operable association with a promoter element of a suitable expression vector. Alternatively, the promoter element can be obtained directly from the targeted gene of interest and the promoter-containing fragment linked upstream from the GFP or luciferase coding sequence to produce chimeric GFP proteins.

Please amend the paragraph beginning on page 120, line 10, through page 121, line 6, as follows:

1. Mutation of GFP surfaces to disrupt multimerization

Figure 5 depicts the three anthozoan fluorescent [protein] proteins for which a crystal structure exists[,]; another is available commercially from Clontech as dsRed (also known as drFP583, as in this alignment) (Wall *et al.* (2000); *Nature Struct. Biol. 7*:1133-1138; Yarbrough *et al.* (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*: 462-467). A dark gray background depicts amino acid conservation, and a light gray background depicts shared physiochemical properties. These crystal structures and biochemical characterization (Baird *et al.* (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*: 11984-11989) show that dsRed exists as an obligate tetramer *in vitro*. Evidence also exists that dsRed multimerizes in living cells (Baird *et al.* (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*: 11984-11989). Sedimentation and native gel electrophoresis studies indicate that *Ptilosarcus* and *Renilla mullerei* GFPs also form tetramers *in vitro* and multimerize *in vivo*. *Ptilosarcus* and *Renilla mullerei* GFPs diverge strongly in amino acid sequence

from dsRed (39% and 38% identical, respectively). Computational polypeptide threading algorithms predict that these GFPs fold into essentially the same structure as dsRed, and also the much more sequence divergent *Aequorea victoria* GFP. *Renilla reniformis* GFP is similarly related in sequence to dsRed, *Ptilosarcus* and *Renilla mullerei* GFPs (37%, 51% and 53% identical, respectively), and thus is extremely likely to form similar multimers. Multimerization is undesirable for many applications that use GFP as the reporting moiety in chimeric protein fusion. Hence muteins in which the capacity to multimerize is reduced are provided. Thus provided are mutations of *Renilla reniformis* GFP that disrupt the formation of GFP multimers. Such mutations may also be effected in the *Ptilosarcus* and *Renilla mullerei* and other GFPs (see Figure 6).

Please amend the paragraph beginning on page 121, line 29, through page 122, line 2, as follows:

Site directed mutagenesis techniques are used to introduce amino acid side chains that <u>are</u> amenable to aqueous solvation, and [at] that significantly alter surface sterochemistry. Disruption of interacting surfaces involves loss-of-function mutagenesis. It is thus contemplated that altering only a few residues, perhaps even one, is sufficient.

Please amend the paragraph on page 123, lines 5-20, as follows:

Among the transgenic plants and animals provided are those that are novelty items, such as animals with eyes or fingernails or tusks or hair [or] that glows fluorescently. Transgenic food animals, such as chickens and cows and pigs are contemplated from which glowing meat and eggs (green eggs and ham) can be obtained; glowing worms can serve as fishing lures. In addition, the *Renilla reniformis* can serve as a reporter to detect that a heterologous gene linked to the GFP gene is incorporated into the animal's genome or becomes part of the genome in some or all cells. The *Renilla reniformis* can similarly be

used as a reporter for gene therapy. The GFP can be introduced into plants to make transgenic ornamental plants that glow, such as orchids and roses and other flowering plants. Also the GFP can be used as a marker in plants, such as by linking it to a promoter, such as Fos that responds to secondary messages to assess signal transduction. The GFP can be linked to adenylcyclase causing the plants to emit different spectral frequencies as the levels of adenylcyclase change.

Please amend the paragraphs beginning on page 126, line 16, through page 127, line 22, as follows:

1. Design of sensors based on BRET

Resonance energy transfer between two chromophores is a quantum mechanical process that is exquisitely sensitive to the distance between the donor and acceptor chromophores and their relative orientation in space (Wu & Brand (1994) *Anal. Biochem. 218*:1-13). Efficiency of energy transfer is inversely proportional to the 6th power of chromophore separation. In practice, the useful distance range is about 10 to [100 A]<u>100 A°</u>, which has made resonance energy transfer a very useful technique for studying the interactions of biological macromolecules. A variety of fluorescence-based FRET biosensors have been constructed, initially employing chemical fluors conjugated to proteins or membrane components, and more recently, using pairs of spectrally distinct GFP mutants (Giuliano & Taylor (1998) *Trends Biotech. 16*:99-146; Tsien (1998) *Annu. Rev. Biochem. 67*:509-44).

Although these genetically encoded GFP [bioluminescence -based] <u>bioluminescence-based</u> biosensors have advantages over less convenient and less precise chemical conjugate-based biosensors, all share a limitation in their design: it is generally difficult to construct a biosensor in which energy transfer is quantitative when the chromophores are in closest apposition. It is almost impossible to arbitrarily manipulate the complex stereochemistry of proteins so that conjugated or intrinsic chromophores are *stably positioned with minimal*

separation and optimal orientation. The efficiency of such biosensors are also often limited by stoichiometric imbalances between resonance energy donor and acceptor; the donor and acceptor macromolecules must be quantitatively complexed to avoid background signal emanating from uncomplexed chromophores. These limitations in general design become important when biosensors must be robust, convenient and cheap. Developing technologies such as high throughput screening for candidate drugs (using high throughput screening (HTS) [protocoals]protocols), biochips and environmental monitoring systems would benefit greatly from modular biosensors where the signal of a rare target "hit" (*e.g.*, complex formation between two polypeptides) is unambiguously (statistically) distinguishable from the huge excess of "non-hits"). Current genetically encoded FRET and bioluminescence-based biosensors display hit signals that very often are less than two-fold greater than non-hit signals, and are at best a few-fold greater (Xu *et al.* (1999) *Proc. Natl. Acad. Sci USA 96*: 151-156; Miyawaki *et al.* (1997) *Nature 388*:882-7).

Please amend the paragraphs beginning on page 130, line 22, through page 131, line 19, as follows:

The BRET sensors can [for]be used for hit identification and downstream evaluation in *in vitro* screening assays in *in vitro* or *in vivo* or *in situ*, including in cultured cells and tissues and animals. The BRET sensors can be created by thermal endpoint-selection, which is suited to DBRET (Disruption-of-BRET) and reduces need for knowledge of target 3D structure and functional dynamics. Existing screening robotics can be used to optimize biosensors. BRET sensors benefit from vast genetic diversity, anthozoans have evolved efficient luciferase-GFP energy transfer systems and the components can be mixed and matched. Highly efficient heterologous luciferases may be substituted for less active luciferases. For example, a copepod luciferase active site can be fused to an anthozoan luciferase GFP-binding domain. There are many diverse coelenterazine-using luciferases.

BRET sensors are modular so that an optimized sensor scaffold may be used with different targets. Also the BRET acceptor may be varied to give shifted emissions, facilitating multiple simultaneous readouts. The anthozoan GFPs can be mutated, [GPFs]GFPs or other proteins can be modified with different chemical fluors, high throughput screening (HTS) fluor-modified FRET acceptors can be adapted, and the BRET donor (luciferase) may be varied, such as by using an Aequorin (Ca++ activated) photoprotein, or a firefly luciferse (requires ATP and a firefly luciferin) to give conditional activation. The sensor scaffold can be incorporated into a variety of immobilization motifs, including free format plates, which can reduce reagent volumes, reusable microtiter plates, miniature columns and biochips. Finally, BRET sensors are inexpensive and reproducible reagents because they can be produced by standardized protein production and can incorporate purification tags. Genetically encoded reporters more reproducible than chemically modified reporters. Linear translation of BRET modules ensures sensor integrity.

Please amend the paragraphs beginning on page 131, line 23, through page 132, line 12, as follows:

EXAMPLE

Specimens of the sea pansy *Renilla reniformis* were collected from inshore waters off the coast of Georgia. To prepare the sea pansies for isolation of mRNA, about 25 or so at time were placed on a large bed of dry ice. They were flipped with a spatula to flip them over to prevent them from freezing. Oddly, the entire animal illuminated when it came in contact with the dry ice. The brightest and greenest were culled, placed in a bag and back into sea water at about 65-70° C for two hours. This process of dry ice, culling and sea water treatment was repeated three [time]times over a 6 hour period. In addition, the process was performed at night. After they were exhausted [were]with the last chilling, the culled animals were frozen solid. A cDNA library was prepared from the frozen animals.

The animals that were selected this way were frozen in liquid nitrogen, and shipped to Stratagene, Inc. (La Jolla, Ca)[.], a commercial vendor whose business includes the construction of custom cDNA libraries under contract to prepare the library. Purified polyA-mRNA was prepared, and a cDNA synthesis reaction was performed, appending a 3' *XhoI* site and a 5' *EcoR*I restriction site to the cDNA. The cDNA was inserted by ligation between the *EcoR*I and *Xho*I sites of the Uni-ZAP Lambda phage cDNA cloning vector.

IN THE CLAIMS:

Please amend claims 19, 26, 40, 47, 53, 58, 64, 67, 70, 73, and 74 as follows:

19. (Amended) The composition of claim 17, wherein the bioluminescence generating system is selected from those isolated from: fireflies, *Mnemiopsis*, *Beroe ovata*, *Aequorea*, *Obelia*, *Vargula*, *Pelagia*, *Renilla*, *Pholas Aristostomias*, *Pachystomias*, *Poricthys*, *Cypridina*, *Aristostomias*, [such] *Pachystomias*, *Malacosteus*, *Gonadostomias*, *Gaussia*, *Watensia*, *Halisturia*, Vampire squid, *Glyphus*, Mycotophids, *Vinciguerria*, *Howella*, *Florenciella*, *Chaudiodus*, *Melanocostus*, Sea Pens, *Chiroteuthis*, *Eucleoteuthis*, *Onychoteuthis*, *Watasenia*, cuttlefish, *Sepiolina*, *Oplophorus*, *Acanthophyra*, *Sergestes*, *Gnathophausia*, *Argyropelecus*, *Yarella*, *Diaphus*, *Gonadostomias* and *Neoscopelus*.

26. (Amended) The combination of claim 24, wherein the component of the bioluminescence generating system comprises a [comprises a] luciferin.

40. (Amended) The nucleic acid construct of claim 39, wherein the *Gaussia* luciferase is a *Gaussia [princepes]*princeps luciferase.

47. (Amended) The construct of claim 38, wherein the encoded luciferase and fluorescent protein comprise a fusion protein.

53. (Amended) The fusion protein of claim 51, wherein the luciferase [and] is a *Renilla reniformis* luciferase.

58. (Amended) The nucleic acid construct of claim [54] 57, comprising a sequence of nucleotides that encodes a ligand binding domain of a target protein.

64. (Amended) A bioluminescence resonance energy transfer (BRET) system, comprising:

(a) a GFP encoded by the nucleic molecule of claim 1;

(b) a luciferase from which the GFP can accept energy when the GFP and luciferase associate; and

(c) a luciferin or other substrate of the luciferase.

67. (Amended) The BRET system of claim 65, wherein a [conformation] conformational change in a modulator causes an increase in the proximity of the luciferase and GFP.

70. (Amended) A microelectronic device, comprising:

a substrate;

a plurality of micro-locations defined on the substrate, wherein each micro-location is for linking a macromolecule;

an independent photodetector integrated at or adjacent to each micro-location and optically coupled to each micro-location, each photodetector being configured to generate a sensed signal responsive to the photons of light emitted at the corresponding micro-location when a light-emitting chemical reaction occurs at that micro-location, each photodetector being independent from the photodetectors optically coupled to the other micro-locations; and

an electronic circuit coupled to each photodetector and configured to read the sensed signal generated by each photodetector and to generate output data signals therefrom that are indicative of the light emitted at each micro-location by the light-emitting chemical reactions, whereby the device detects photons of light emitted by light-emitting chemical reactions, wherein:

each micro-location is defined by a portion of the surface; and

the micro-locations defined on the substrate each comprise a [components] component of a bioluminescence generating system and a green

fluorescent protein of claim 1, whereby photons of light are emitted when a reaction takes place at that micro-location.

73. (Amended) The device of claim 71, wherein the bioluminescence generating system comprises [s] a *Renilla reniformis* luciferase.

74. (Amended) A method of detecting and identifying analytes in a biological sample, comprising:

providing the microelectronic device of claim 70;

attaching a macromolecule or plurality of different macromolecules to the surface at each micro-location on the device, wherein macromolecule is specific for binding to selected analyte that may be present in the biological sample;

contacting the sample with the surface of the microelectronic device, whereby any of the selected analytes that are present in the sample bind to the macromolecule attached to the surface at each micro-location;

exposing the surface of the microelectronic device to a second macromolecule or plurality thereof [bind] bound to the selected analyte already bound to the first macromolecule at each micro-location, wherein the second macromolecule comprises a component of a bioluminescence generating reaction;

initiating the bioluminescence generating reaction by contacting the surface of the device with the remaining components of the bioluminescence generating reaction, wherein the wavelength of the resulting light is shifted by the *Renilla reniformis* GFP; and

detecting photons of light emitted by the GFP using a photodetector optically coupled to each micro-location, each photodetector generating a sensed signal representative of the bioluminescence generation at the respective micro-location.